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Reprinted from SCIENCE, August 14, 1953, Vol. 118, No. 3059, pages 169–175.

Sex in Bacteria: Genetic Studies, 1945–1952[1]

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FOR MANY YEARS bacteria were considered biologically exceptional organisms with no genes, nuclei, or sex, although the recognition of their biochemical similarities to other forms of life constituted one of the main foundations of comparative biochemistry. Over the last decade evidence has accumulated which has led to the satisfying conclusion that bacteria are not biologically unique but possess genetic and behavior systems more or less analogous to those of other forms, including nuclei, genes, and in certain instances even true sexual mechanisms for recombination of unit characters.

Historically, this change in our thinking in regard to bacteria stems from the pioneer concepts of Lwoff (1938) and Knight (1936) relating the nutritional requirements of microorganisms to an evolutionary loss of synthetic abilities. If such losses in microorganisms were based on mutation and selection as required by modern concepts of evolution, the capacities for synthesis of essential nutrilites in microorganisms should be determined by genes, which should be subject to mutation, as are most genes in other organisms. Such considerations led Beadle and Tatum (1941) to the successful production by irradiation of nutritionally deficient or biochemical mutants in the heterothallic ascomycete *Neurospora,* and to the establishment of the genic basis of biochemical reactions leading to the synthesis of amino acids and vitamins. This relation has since been amply substantiated and extended by further work with *Neurospora* and other sexual microorganisms (cf. Tatum and Perkins, 1950).

The next step in the evolution of our concepts of bacterial genetics was the experimental application of the *Neurospora* techniques to the production of biochemical mutants in these simpler organisms. The first nutritionally deficient (auxotrophic) mutants were produced in 1944 by x-ray treatment of *Escherichia coli* and *Acetobacter melanogenum* (Gray and Tatum, 1944; Roepke *et al.,* 1944). Subsequently, auxotrophic mutants have been obtained in almost every species of bacteria investigated (see Tatum, 1946; Tatum and Perkins, 1950). In addition to mutations to growth-factor dependence and reverse mutation to growth-factor independence, other types of mutant characters have been obtained, thoroughly investigated, and proved extremely valuable. These include such characters as virus resistance, antibiotic resistance, and capacity for sugar utilization.

The first auxotrophic mutants were obtained by the tedious and laborious process of plating out irradiated cells on fully supplemented medium, and then isolating individual colonies which were subsequently examined for failure to grow in the simple synthetic medium adequate for the original stock (minimal medium). The specific requirements of these deficient clones were then determined by systematic supplementation of the minimal medium with known growth-factors.

Later improvements in techniques have eliminated much of the labor involved in isolating and testing mutants of bacteria, particularly *E. coli.* These include (cf. Lederberg 1950, 1951a) the layer-plate technique in which only presumptive mutants are isolated for further testing, and the penicillin method in which non-mutants are actively eliminated, leaving mutant cells which form colonies after removal of the antibiotic and suitable supplementation of the medium. A further modification of this method, using solid medium and penicillinase, permits easy isolation of any desired type of auxotroph (Adelberg and Myers, 1952), and the isolation and testing of strains has been still further simplified by the replica plating method (Lederberg and Lederberg, 1952) which in a single operation permits transferring all colonies on a plate to a number of other plates with different supplements.

By all available criteria it is now generally accepted that most, if not all, characteristics of bacteria are controlled by hereditary units, and that these hereditary units in bacteria are analogous with genes in classically sexual organisms in the independence and randomness of their mutation, the effect of physical and chemical agents on their mutation frequency, and qualitatively in the types of biochemical and enzymatic effects of their mutation (cf. Tatum and Perkins, 1950).

After establishment of this functional analogy between genes of bacteria and those of sexual forms, the next step logically was to ask if the analogy could be carried further, and if a mode of inheritance of bacterial characters similar to the Mendelian process in higher types could be detected. Although a number of workers had looked for character recombination in bacteria, and other microorganisms, even as early as 1908 (Browning, 1908; for other important references cf. Tatum and Lederberg, 1947), in most cases the biological materials available were not adequate for a definitive experimental test, although some sug-

[1] This paper was prepared in connection with a Symposium on Sex in Microorganisms, held in December 1951 and to be published in a forthcoming volume by the AAAS.

[2] Paper No. 498 of the Department of Genetics. This work has been supported by research grants (E72) from the National Microbiological Institute, National Institutes of Health, Public Health Service, and from the Research Committee, Graduate School, University of Wisconsin, with funds provided by the Wisconsin Alumni Research Foundation.

gestive evidence of a circumstantial nature has been obtained. The definitive nature of auxotrophic mutants and the relative ease of their isolation and diagnosis provided ideal material for testing the possibility of recombination of hereditary units in bacteria.

The independent occurrence and expression of auxotrophic mutations in *E. coli* permitted building up multiple mutant stocks of *E. coli* strain K-12 with several deficiencies by successive mutational treatment (Tatum, 1945). In this way, for example, cultures Y-10, requiring threonine, leucine, and thiamin, and 58-161, requiring biotin and methionine, were obtained. For simplicity in considering its capacity for synthesis of the factors concerned, strain 58-161 can be represented as biotin– methionine– threonine+ leucine+ thiamin+ (*B– M– T+ L+ B_1+*), while strain Y-10 would similarly be represented as *B+ M+ T– L– B_1–*. In this representation, in analogy with other organisms such as *Neurospora*, the genes determining alternative characters (*B+* and *B–* for example) are considered allelic.

Accordingly, a sexual process in a mixed culture of these two strains would involve reshuffling the indicated alleles at these five loci. If this were at random, any recombination might be expected, and might have been looked for. However, recombination to give a nutritionally independent type (prototroph) would be most easily detected, since it alone would grow on minimal medium, whereas any dependent types, including both parental strains, would not.

Experimental tests were carried out (Tatum and Lederberg, 1947) by growing the two strains either separately or together in complete media, then centrifuging out the cells, washing repeatedly to remove growth-factors, and plating mixtures of the washed cells into minimal agar. The results were striking in that about 100 colonies developed for each 10^9 cells examined, and on reisolation and purification these maintained their prototrophic character. Similarly treated single cultures of each strain gave no colonies on the minimal medium. This would be expected on the basis of the low frequency of mutation of each character to independence (*ca.* 1 in 10^7 cells) since the derivation of a prototroph from a triply deficient strain would then occur with a frequency of 1 in 10^{21} cells.

The simplest explanation for these results therefore appeared to be that gene recombination took place to give the prototroph *B+ M+ T+ L+ B_1+*, as shown in Fig. 1. Other possibilities, such as association of cells, or the formation of unsegregated diploid, or of heterocaryotic cells were made unlikely by various experimental tests which established the homogeneity and uniqueness of the derived prototrophs (cf. Tatum and Lederberg, 1947).

The only alternative to a sexual recombination seemed therefore a type of unilateral change by a noncellular transforming principle similar to that involved in induced changes of type in the pneumococcus (Austrian, 1952). Two lines of evidence made this improbable. First, cell-free filtrates gave no prototrophs and direct contact of the cells themselves seemed essential, as shown by growing the two types separated by an ultra-fine sintered glass bacterial filter (Davis, 1950). Second, the successful recovery of most of the possible recombination types in later

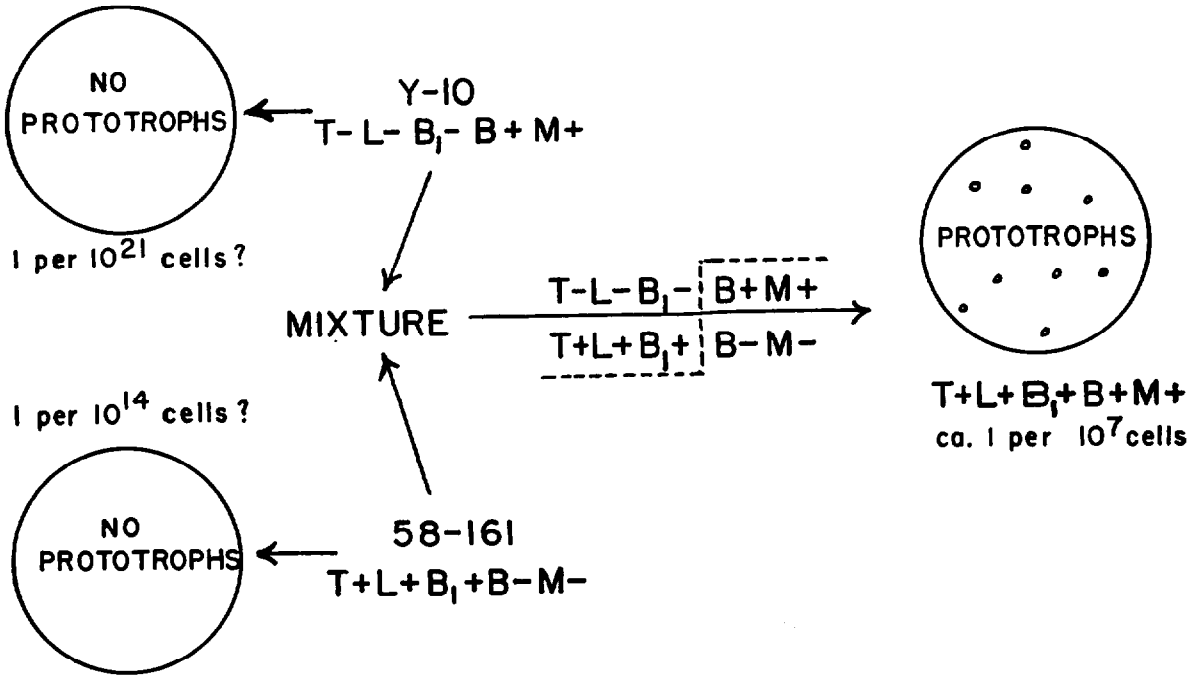

FIG. 1. Diagrammatic representation of recombination in prototrophs.

crosses involving a much greater variety of characters, including resistance to antibiotics and viruses and sugar fermentation characters, would necessitate simultaneous transformation in different directions for different characters, and in both directions in different cells.

Thus the results of experiments of the type described above are satisfactorily explained only as resulting from a sexual mating process, followed by reassortment or segregation of genetic material. Repetition and extension of these experiments in a considerable number of laboratories during the past five years have amply confirmed the reality of the essential phenomenon and the validity of this conclusion.

These experiments have added considerable information about environmental factors affecting the recombination process, and support the concept that direct cell contact is necessary for the sexual process. Some of the strongest support comes from the demonstration by Nelson (1951) that recombination behaves as a bimolecular reaction, as if factors such as relative and absolute concentrations of the two types of cells which would affect the frequency of contact of appropriate cells, similarly affect frequency of recombination. The experiments of Davis (1950), showing the need for cell contact, and in a more positive sense the production of a genetically diploid cell (Lederberg, 1949) likewise support the postulated occurrence of a cell to cell sexual process in *E. coli* K-12.

The intimate details of mating are still obscure. Owing to its infrequency we have been discouraged (until very recently) from any serious attempts to detect its morphological basis, and were obliged to be content with genetic inferences. E. Klieneberger-Nobel, of the Lister Institute, London, England, has made a most painstaking study of mating cultures of *E. coli* K-12 (unpublished work, quoted by her kind permission). Although she occasionally observed what appeared to be stages in the abortive development of L-forms (Klieneberger-Nobel, 1951), she was unable to correlate them in any way with recombination. The only conclusion that is warranted is that recombination in *E. coli* does not involve spectacular formations, such as have been observed and speculated about in many bacteria. The possibility of a mating process involving a rapid conjugation and separation of the parent cells, without the intervention of special gamete or zygote structures, has not been excluded and is perhaps most likely.

The recently reported work of Hayes may throw further light on the conjugation process. Working with a single pair of K-12 stocks, Hayes (1952a) found that streptomycin treatment sterilized one (58-161) without affecting its recombination potency, but completely abolished recombination potency of the other (W677), and he postulated a unidirectional transfer of metabolically inert genic material from 58-161 to W677. The results of later studies (Hayes, 1952b) using ultra-violet irradiation for cell inactivation and stimulation of recombination (Clark *et al.*, 1950) were consistent with this hypothesis. He has suggested that the recombination may involve only a limited transfer of genic material, through a process which may not require the participation of two intact cells.

Granted the ability of *E. coli* K-12 to undergo a sexual recombination of genetic characters analogous to that found in other organisms, even if the morphological basis is still obscure, can the analogy be carried further? What evidence exists bearing on a chromosomal organization of the genes in *E. coli*? In all organisms that have been adequately studied, the genes are arranged in a linear order on chromosomes, whose distribution at cell division and during the formation of gametes follows very precise laws. It is difficult, in fact, to conceive of any other arrangement of the genes that would permit their regular and orderly distribution to the products of each cell division, without an uneconomical redundancy of the genetic factors for different traits. But aside from these speculations, there is considerable experimental evidence that the genes of *E. coli* are organized in linear order on one or more chromosomes (Lederberg, 1947; Rothfels, 1952; Lederberg *et al.*, 1951).

In the crosses mentioned so far, all the differences between the parents are directly involved in the selection of recombinants, so that we had no opportunity to investigate the segregation of factors whose expression is not enforced by the selective method. A number of mutant characters have been discovered, however, which are indifferent to plating on minimal medium. They include differences in the fermentation of various sugars, resistance to antibiotics, and resistance to phages. Such characters will be called unselected, since their segregation is regulated by the internal mechanism of recombination rather than the exigencies of the technique.

The first unselected marker to be used in our experiments was resistance to phage T1 (Tatum and Lederberg, 1947). According to conventions, resistance and sensitivity are symbolized as $T1^r$ and $T1^s$, respectively. $T1^r$ is a specially convenient marker, as it can be produced in any stock by the selection of spontaneous mutants with T1. Before they can be used in these experiments, such stocks must be carefully purified and, as for any marker, the stability and reproducible scoring of the mutation must be verified. A variety of crosses was carried out in which one parent was $T1^r$, the other $T1^s$. In each case we found a segregation for this marker, i.e., some of the prototrophs displayed the $T1^r$ trait, from one parent, and others $T1^s$ from the other. In control crosses, $T1^s \times T1^s$ gave only $T1^s$, and $T1^r \times T1^r$ gave only $T1^r$. Such a segregation in the first filial generation, the f-1, indicates a haplobiontic life cycle, similar to that of many unicellular organisms.

If an unselected marker were associated with one chromosome independent of others carrying the selected, nutritional mutations, the f-1 should show a mendelian ratio of 1:1. Different ratios were ob-

served for each of the markers tested, the first evidence of linkage. The observed frequencies varied from one marker to another, and with a given marker, from one parental combination to another. In a given cross, however, the f-1 segregation ratios have been as reproducible as in any genetic material, which is to say they are subject mainly to sampling error.

A simple test of the significance of f-1 ratios can be made by reverse crosses, whereby a marker is introduced first in one, then in the other parent. For example, *BM– $V1^r$ × TL– $V1^s$* is compared with *BM– $V1^s$ × TL– $V1^r$*. About 70% of the prototrophs from the first cross are $V1^r$. In the second cross, about 30% are $V1^r$, i.e., this ratio is inverted. The same result has been obtained in many reverse crosses involving different parental lines, and different markers and combinations of markers. It shows that the f-1 ratios have nothing to do with the physiological effects of the markers, but that they are due entirely to the mechanics of segregation. It also shows that dominance plays no role, and more generally that a genetic particle controlling each observed trait has been segregated, and is represented only once in the genotype of the recombinant cell.

By compounding elementary principles, genetic maps of *E. coli* can be constructed from segregation data involving numerous unselected markers (Cavalli, 1950; Newcombe and Nyholm, 1950; Rothfels, 1952; Lederberg *et al.*, 1951). By using other methods, the auxotroph mutations can be relieved of their burden of the selection of recombinants, and thus handled as unselected markers also. About half of the known markers of *E. coli* K-12 have been satisfactorily located in a single linear linkage group. Other markers have displayed a more confusing behavior which does not fit any scheme very satisfactorily, but is probably a result of rather complex chromosomal aberrations, for which there is independent evidence from the study of exceptional diploids (*v. infra*). It was long thought that *E. coli* had only one chromosome, but more recent evidence points to at least two, the segregation of which is not, however, entirely independent for secondary reasons (Fried and Lederberg, 1952). Cytological observations on haploid K-12 cultures have been interpreted by DeLamater (1952) as signifying three chromosomes, but further work is needed for the detailed concordance of genetic with cytological findings. Cytological study of *E. coli* has so far been confined to vegetative cells, whereas the genetic studies deal principally with segregation at meiosis.

The difficulties, briefly mentioned, in the segregation of certain misbehaving markers might appear to be fatal to a straightforward sexual interpretation of recombination except for the confirmatory evidence provided by exceptional diploid cultures. In ordinary crossings, the diploid condition has been inferred from its consequences of recombination and segregation, but is not directly observed. In 1946–47, many unsuccessful attempts were made to secure artificial diploids with agents such as camphor, acenaphthene, colchicine, and heat shocks, which have been used for other organisms (cf. Roper, 1952). More recently, however, a mutation, *Het,* occurred in one of our stocks which serves the same purpose (Lederberg *et al.,* 1951). Little is known of the action or genetic transmission of *Het,* but when it is present in one or both parents of a cross, several per cent of the prototrophs prove to be persistent heterozygotes. These heterozygous cultures continually segregate the alternative markers brought in by the parents. Thus diploid cells heterozygous for lactose fermentation, *Lac+/Lac–,* produce mosaic colonies on an indicator medium, as shown in Fig. 2. The dark or "+" sectors consist of still heterozygous cells, *Lac+/Lac–,* and of *Lac+* haploid segregants; the light or "–" sectors are *Lac–* haploid segregants. On complete medium, the faster-growing haploid cells soon outstrip the original diploids, but segregation can be effectively prevented on a minimal medium owing to the nutritional requirements of the haploid components. Single cell studies (Zelle and Lederberg, 1951) have verified that genetic factors from two parents have converged to a single hybrid cell, the essence of sexuality.

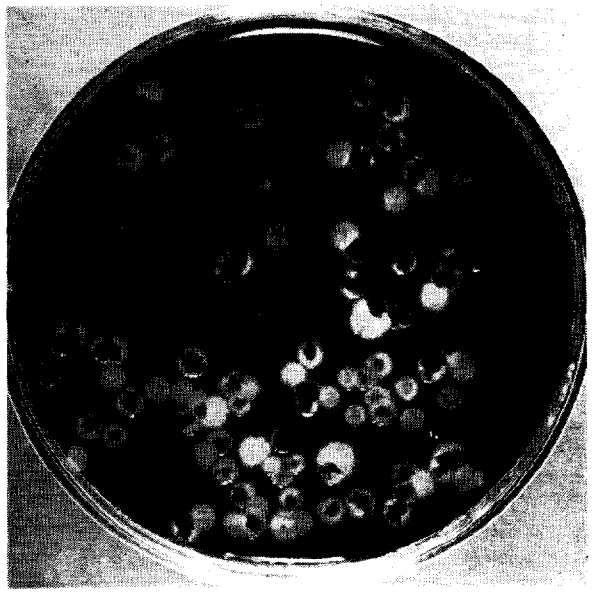

FIG. 2. Segregating diploid culture plated on indicator agar, showing variegated colonies.

Haploid and diploid cultures have been studied cytologically, especially for comparisons of their nuclear structure, by means of the Piekarski-Robinow technique (osmic fixation; HCl hydrolysis; Giemsa stain; mount in Abopon). This method gives brilliant nuclear preparations, but *E. coli* appears to be technically unsuitable for unequivocal demonstration of mitotic figures, as claimed in the pioneering and provocative work of DeLamater and his associates (1951). Although nuclear aggregates that are very suggestive of mitotic metaphases and anaphases can be found with a brief search, definitive interpretations of *E. coli* cytology depend for the most part on the

validity of the conclusions that have been drawn from technically superior material. It is difficult for a geneticist to imagine how bacteria could get along without some sort of mitotic process, but its details require critical, and objective definition. The comparisons of haploid and diploid *E. coli* have revealed consistent and unequivocal differences, as shown in photographs published elsewhere (Lederberg *et al.*, 1951). The determination whether the diploids show a doubling of the chromosome number is not yet subject to independent, objective verification.

The correlation of genetic heterozygosity with nuclear complexity is only a small step in the direction of a bacterial cytogenetics. It has been furthered by Witkin's studies, in which the segregation of mutant genes during fission has been correlated with the nuclear plurality of the bacterial cells at the time the mutations were induced (Witkin, 1951). These observations do accord, however, with a chromosomal theory of inheritance and sexuality in *E. coli.*

The work cited so far has been done with derivatives of a single strain, K-12, of *Escherichia coli.* A few early attempts to duplicate genetic recombination in other *E. coli* strains popular in genetic work were quite unsuccessful. Cavalli and Heslot (1949) discovered a culture in the British Type Culture Collection, NTCC 123, that was fertile with K-12, but a special screening method had to be developed before many new strains could be effectively studied (Lederberg, 1951a). Of nearly 2,000 independent isolations of *E. coli* from various sources, over fifty have proven to be cross-fertile with K-12, and so far as has been tested, with each other. All of the new strains conform to the type *E. coli,* except for an occasional minor deviation, but are otherwise as heterogeneous as any sample of strains. They are serologically quite diverse: an immunogenetic study has been initiated which has so far put the antigens of *E. coli* on the same basis as the mammalian blood groups.

One important reason for undertaking this study of new strains was to investigate the sexual compatibility relations of *E. coli.* Until recently, several lines of evidence conspired to substantiate the idea that *E. coli* K-12 was homothallic. The crossable strains are all derived from a single pure culture. Several workers had suggested that a mating-type system might be obscured by mutations from one mating type to the other, as occurs in certain yeasts. However, this hypothesis was rejected because no segregation of mating preferences was observed from heterozygous diploids, as would have been expected from a heterothallic mating. It has since been discovered that a unique compatibility mechanism does operate in *Escherichia coli* (Lederberg, Cavalli, and Lederberg, 1952). The involved history of this discovery must be detailed elsewhere, and only the general conclusions can be given here.

Wild type K-12 carries a hereditary factor, *F+*, which is required for mating. Similarly, most of the auxotrophic mutants of K-12 are *F+*, and therefore mutually compatible, but the much used line descended from the threonine-leucine mutant, 679-680 (Tatum, 1945), is *F−*. Because most of the other tester cultures are *F+*, however, the *F−* "mutation" was not detected in earlier experiments. The empirical definition of *F−* is that crosses of two *F−* parents are completely sterile, although comparable crosses in which one or both parents is *F+* are productive. The self-incompatibility of *F−* has been detected in two ways: sublines of 679–680 are mutually incompatible, and new occurrences of the *F−* "mutation" have been discovered which are incompatible with 679–680. The *F−* "mutation" is given in inverted commas because its inheritance and transmission set it apart from all of the other markers so far studied.

All the progeny of crosses within strain K-12 are *F+*, whether the parents were $F- \times F+$ or $F+ \times F+$ [$F- \times F-$ cannot, of course, be tested]. This was explained by the finding that F+ was contagious, that is that growing *F−* cells in mixture with *F+* resulted in many of the former (identified by other genetic markers) becoming permanently *F+*. As many as 50 per cent of the originally *F−* cells may become *F+* by this conditioning process within a few hours. "*F+*" is therefore tentatively regarded as an infective, virus-like agent, but this has not yet been confirmed by the isolation of "*F+*" in a cell-free preparation. The transmission of *F+* is not accompanied by the transfer of any other marker, so far as is known.

The virus-like properties of the compatibility factor have led to some speculation on its relationship to a virus known to be present in *E. coli* K-12, the latent bacteriophage λ. This question has been studied in some detail, and it can be asserted that λ is not related in any way to genetic recombination or to the sexual compatibility mechanism. Non-lysogenic, i.e., λ-free cultures are fully compatible in sexual recombination, and the transfer of *F+* is independent of the transfer of λ, which unlike *F+*, can readily be obtained in cell-free filtrates. Genetic studies of lysogenicity (Lederberg and Lederberg, 1953) have, however, demonstrated a close relationship between this latent virus and the chromosomes of the bacterial host.

The details of the compatibility system are being studied at the present time. It has been noted that $F+ \times F+$ crosses tend to be considerably less productive than comparable $F+ \times F-$. Many of the results are consistent with the concept of relative sexuality as noted in many algae and fungi. That is to say, different cultures can be arranged in sequence of relative potencies, such that the productivity of a cross will be related to the difference in potency of the two parents. In *E. coli* K-12, the relative potency can be controlled both by environmental variations, and by genotypic effects. Within K-12, differences in the *F+* agent itself have not been found. However, the *F+* state as conditioned by some other wild-type strains appears to be unstable in K-12 cells, suggesting the possibility of genetic differences in the presumed agent itself.

The genetic basis of the observed "mutations" to *F*– in strain K-12 is not known, and these have not been experimentally reproducible. Many wild type strains are *F*– (i.e., non-infective) but retain their compatibility status, so that it is impossible to generalize on the causes of sterility or compatibility in the species *E. coli* taken as a whole.

In the absence of direct morphological evidence of sexual fusions, the principal alternative explanation for genetic recombination in *E. coli* has been "transformation" or "transduction." The biology of bacterial transformations is not very well understood (Ephrussi-Taylor, 1951; Austrian, 1952); in many ways it may be constructive to regard them as a limited form of hybridization. The chief distinction between transduction and sexual recombination is that the former seems to involve only a very small part of the whole genotype of the bacterium at each transfer (as in the capsular transformations in the pneumococcus), whereas sexual reproduction allows reassortment of the entire genotype of each parent, as in *E. coli.* The former seems to be correlated with an active unit that is morphologically and chemically much simpler than the intact cell; in *E. coli,* no unit other than the cell has been shown to be active in recombination.

A search for recombination in *Salmonella typhimurium,* a species distantly related to *E. coli,* has led to the discovery of another mode of transduction. In this system occasional bacteriophage particles appear to become fortuitously contaminated with genetic fragments from the host cells on which they are grown, and to be able to transduce these fragments to new cells which they may invade without killing them. The fragments retain their activity, and somehow enter the genetic organization of the new host. In this way, for example, flagellar antigenic traits from *S. typhimurium* may be introduced into cells of *S. typhi* to give a hybrid serotype or species not previously described (Zinder and Lederberg, 1952). The possibilities that sexual recombination may occur in *Salmonella,* or that a genetic transduction may be found in *E. coli* are not unlikely, in view of the taxonomic relationship of these bacteria. It is from a superposition of these phenomena in a single species that we may expect to learn the most about each of them. At present, however, they appear to be quite distinct.

To sum up the present status of our knowledge of sexuality in *E. coli,* it has been shown that in mixed cultures under suitable conditions a small but significant number of cells of certain strains of this organism undergo a process of recombination of genes governing a wide variety of characters. This process apparently involves a cell-to-cell contact, and presumptively copulation, or conjugation, with zygote formation. Analysis of the recombination products indicates that the genes are present in linear order on one or more chromosomes. In essence, then, certain strains of *E. coli,* especially K-12, are capable of a sexual process, analogous in so far as it has yet been analyzed to that of other organisms.

It is to be hoped and expected that sexual phenomena will not be limited to *E. coli* among the bacteria. It is likewise to be hoped that the significance of suggestive cytological phenomena in bacteria such as apparent conjugation tubes (DeLamater, 1951), star-body formation (Braun and Elrod, 1946), filterable L-forms (Klieneberger-Nobel, 1951) and large bodies (Dienes, 1946; Stempen and Hutchinson, 1951), can be further evaluated by genetic analysis involving the recombination and tracing of suitable genetic markers.

The future also holds not only promise of correlation of genetic and morphological aspects of conjugation and meiosis in *E. coli,* but the even more exciting prospects of discovering, elucidating, and correlating different modifications of sexuality and transfer of genetic material in microorganisms. With a clearer understanding of these relationships, the bacteria may be expected to occupy an increasingly important place in the study of the comparative biology of sex.

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